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(71) Applicant: **THE PROCTER & GAMBLE COMPANY** [US/US]; One Procter & Gamble Plaza, Cincinnati, OH 45202 (US).

(72) Inventors: **DEGENHARDT, Charles, Raymond**; 10555 Wellingwood Court, Cincinnati, OH 45240 (US). **GRAYLING, Rowan, Andrew**; 9855 Hayfield Court, Loveland, OH 45140 (US). **DILLE, Christopher, Andrew**; 325 Timberlake Avenue, Erlanger, KY 41018 (US). **TANSKY, Cheryl, Sue**; 1440 W. Kemper Road, #904, Cincinnati, OH 45240 (US).

(74) Agents: **REED, T., David** et al.; The Procter & Gamble Company, 5299 Spring Grove Avenue, Cincinnati, OH 45217-1087 (US).

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(54) Title: COMPOUNDS, COMPOSITIONS, AND METHODS FOR CONTROLLING BIOFILMS

(57) Abstract: The present invention provides nitrogen heterocyclic compounds, compositions, and methods for controlling biofilms, i.e., disrupting biofilms, preventing biofilm formation, enhancing biofilms, or modifying biofilms. Methods for screening test compounds for control of biofilms and devices for use therein are also provided.

COMPOUNDS, COMPOSITIONS, AND METHODS FOR CONTROLLING BIOFILMS

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] The present application claims the benefit of USSN 60/287,138, filed April 27, 2001.

FIELD OF INVENTION

[0002] This invention relates to compounds, compositions, and methods for controlling biofilms, i.e., disrupting biofilms, preventing biofilm formation, enhancing biofilms, or modifying biofilms. Methods for screening test compounds for control of biofilms and devices for use therein are also provided.

BACKGROUND OF THE INVENTION

[0003] Biofilms are mucilaginous communities of microorganisms such as bacteria, archaea, fungi, molds, algae or protozoa or mixtures thereof that grow on various surfaces (see *Nature*, vol. 408, pp. 284-286, 16 November 2000). Biofilms form when microorganisms establish themselves on a surface and activate genes involved in producing a matrix that includes polysaccharides. This matrix may provide protection of biofilm bacteria from biocides.

[0004] Molecules called quorum-sensing signals help trigger and coordinate part of the process of forming a biofilm. Bacteria constantly secrete low levels of the signals and sense them either through receptors on their surfaces, or internally. The receptors trigger behavioral changes when there are enough bacteria to allow the signals' concentrations to achieve a critical threshold. Once this occurs, bacteria respond by adopting communal behavior, such as forming a biofilm, and in the case of pathogenic bacteria, deploying virulence factors such as toxins. In addition to communicating with members of their own species, bacteria also conduct inter-species communications, such that a biofilm may contain more than one species of bacteria.

BRIEF DESCRIPTION OF THE DRAWINGS

[0011] FIG. 1 is a side cross sectional view of the plastic tube-based biofilm growth and test method invention described in Assay Reference Example 4.

[0012] FIG. 2a is a side cross sectional view of one embodiment of the growth chamber array-based biofilm growth and test method invention described in Assay Reference Example 5.

[0013] FIG. 2b is a side cross sectional view of an alternative embodiment of the growth chamber array-based biofilm growth and test method invention described in Assay Reference Example 6.

[0014] FIG. 3 is a top view of one of the two lids of the growth chamber array-based biofilm growth and test method invention described in Assay Reference Examples 5 and 6.

[0015] FIG. 4 is a top view of the middle piece of the growth chamber array-based biofilm growth and test method invention described in Assay Reference Examples 5 and 6.

DETAILED DESCRIPTION OF THE INVENTION

[0016] The present invention provides compounds and methods for controlling biofilms on a substrate surface. The invention also provides methods for screening test compounds for activity in controlling biofilms, and apparatuses for testing biofilm control on substrate surfaces. "Biofilm" means a mucilaginous community of microorganisms, such as, for example, bacteria, archaea, fungi, molds, algae or protozoa that can grow on various substrates. A biofilm can comprise one or more than one species. "Substrate" means any surface on which a biofilm can form or has formed. Substrate includes, but is not limited to, hard or soft surfaces such as polymers, plastics, tubing, ceramics, metals, glass, hydroxyapatite, skin, bone, or tissues.

[0017] "Controlling biofilms" means herein to disrupt a biofilm and/or prevent biofilm formation, to enhance formation and/or growth of a biofilm, or to modify a biofilm. The invention provides nitrogen heterocyclic compounds having structure A or structure B for controlling biofilms, or a combination thereof, a salt thereof, or a stereoisomer thereof. A method of controlling biofilm on a substrate comprises contacting the substrate with a compound having

[0020] New compounds having structure A or B as set forth infra, or a salt or a stereoisomer thereof, or compositions thereof, are also provided by the present invention. An embodiment of the invention provides compounds having structure A when n is 1, R is H, alkyl, acyl, or alkoxycarbonyl; and R_1 and R_2 are independently H, C_6-C_{20} alkyl, or alkenyl; and wherein when R is H, one of R_1 and R_2 is other than H. Such compounds are synthesized as shown, for example, in synthesis examples 1-3 herein. One of skill in the art in light of the examples herein would be able to synthesize compounds having related substituents. Compounds in which R_1 and R_2 are independently alkenyl are prepared as in example 2 using the appropriate alkenyl amine in place of n-decylamine. Compounds in which R is alkyl are prepared as in example 2 from the appropriate 1-alkyl-2-azetidinecarboxylic acid, the synthesis of which is described by Cromwell *et al.* in *J. Heterocyclic Chem.* (1968), 5(2), 309-311. Compounds in which R is acyl are prepared by reacting the appropriate 2-azetidinecarboxylic acid amide with an acid chloride in a manner similar to example 9.

[0021] A further embodiment of the invention provides new compounds having structure A when n is 3, R is H, alkyl, acyl, or alkoxycarbonyl; and R_1 and R_2 are independently H; C_5-C_{20} alkyl, or alkenyl; and wherein when R is H, one of R_1 and R_2 is other than H. Such compounds are synthesized as shown, for example, in synthesis examples 7-9 herein. One of skill in the art in light of the examples herein would be able to synthesize compounds having related substituents. Compounds in which R_1 and R_2 are independently alkenyl are prepared as in example 7 using the appropriate alkenyl amine in place of n-octylamine. Compounds in which R is alkyl are prepared as in example 7 from the appropriate 1-alkyl-2-piperidinecarboxylic acid, which is available through the reductive alkylation of 2-piperidinecarboylic acid as described by Hu *et al.* in WO 9943658.

[0022] A further embodiment of the invention provides new compounds having structure A when n is 4, R is H, alkyl, acyl, or alkoxycarbonyl; and R_1 and R_2 are independently H; C_5-C_{20} alkyl, or alkenyl. Such compounds are synthesized as shown, for example, in synthesis examples 10 and 11 herein. One of skill in the art in light of the examples herein would be able to synthesize compounds having related substituents. Compounds in which R is acyl are prepared by reacting the appropriate 2-azepanecarboxylic acid amide with an acid chloride in a manner similar to example 9. Compounds in which R is alkyl are prepared as in example 10 from the appropriate 1-alkyl-2-azepanecarboxylic acid, which is available through the reductive alkylation of 2-

A change in the amount of biofilm present as a result of a treatment may result from an effect on the exopolysaccharide matrix of biofilm or an effect on a microorganism within the biofilm, or an effect on the relationship therebetween. Not wanting to be bound by theory, the present inventors believe the nitrogen heterocyclic molecules of the present invention may be mimicking, interfering with or modifying an action of quorum sensing molecules in biofilm communication. The present compounds may also modify a biofilm by, for example, decreasing toxin production, decreasing virulence, by interfering with signal molecules, or by increasing levels of an enzyme that is produced by the biofilm, for example, in a bioreactor.

[0028] A "test compound" is a candidate compound for screening for activity in controlling biofilms. The examples herein test biofilm dispersion and formation in bacterial species that are relevant to consumer and medical environments, and that are broadly representative of bacterial cell wall type. *Pseudomonas* species are tested since these are widespread in biofilms that form in high humidity environments, such as those present in and around showers, toilets, sinks, and drains, and since these are representative gram negative bacteria. *Staphylococcus epidermidis* is tested since it is commonly found on human skin and since it is a representative gram positive bacterium. Both *P. aeruginosa* and *S. epidermidis* are medically important, being opportunistic pathogens and frequent causative agents in hospital-acquired infections, where growth of these organisms in a biofilm state is thought to be important. In addition, these three organisms represent a range of difficulty in treating biofilms, *P. aeruginosa* being the most difficult to control. Screening approaches described herein are also applicable to other bacteria and fungi, including *Staphylococcus aureus*, *Candida albicans*, or *Malassezia furfur*, for example.

[0029] Measurement of biofilm growth herein uses crystal violet as a quantitative, total biofilm staining dye that stains both cells and extracellular polysaccharide, to rapidly identify compounds that are active in controlling biofilms of *P. aeruginosa*, *P. fluorescens*, and *S. epidermidis*. Further dyes or assays also may be used for quantification of biofilms in a similar manner, including but not limited to polysaccharide stains, DNA stains, chemical or biochemical assays, enzyme assays, or physical methods. Such methods are generally destructive to the cells contained within the biofilm.

[0030] Non-destructive alternative methods may also be used for quantification of extent of compound activity in controlling biofilms. Cells may be released intact from biofilms by physical, chemical, or biological methods such as scraping, sonication, agitation in buffer ('stomaching'),

Use of the base in this dispersion assay boosts the sensitivity of the assay as compared to an assay lacking the step of adding a base.

[0035] In an embodiment of the method of controlling biofilm on a substrate, the compound has structure A and n is 1; where controlling is enhancement of biofilm formation or enhancement of existing biofilm, R is alkoxycarbonyl, R_1 is H, and R_2 is C_{10}-C_{12} alkyl; and where controlling is prevention of biofilm formation or dispersion of existing biofilm, R and R_1 are H, and R_2 is C_6-C_{12} alkyl.

[0036] In another embodiment of the method of controlling biofilm on a substrate, the compound has structure A and n is 2; where controlling is enhancement of biofilm formation or enhancement of existing biofilm, R is alkoxycarbonyl or acyl, R_1 is H, and R_2 is C_6-C_{12} alkyl; and where controlling is prevention of biofilm formation or dispersion of existing biofilm, R and R_1 are H, and R_2 is C_{10}-C_{12} alkyl. In another embodiment, R is H, R_1 is H, and R_2 is C_{10} alkyl.

[0037] In another embodiment of the method of controlling biofilm on a substrate, the compound has structure A and n is 3; alternatively, R is H or alkoxycarbonyl, R_1 is H, and R_2 is C_8 alkyl; and wherein controlling is enhancement of formation of biofilm, R is alkoxycarbonyl, acyl, or H, R_1 is H, and R_2 is C_8 alkyl.

[0038] In another embodiment of the method of controlling biofilm on a substrate, the compound has structure A and n is 4.

[0039] In another embodiment of the method of controlling biofilm on a substrate, the compound has structure B and n and m are 1, 3 or 4; alternatively, n and m are 2, and preferably, X is C_{12} alkyl, and R_1, R_2, R_3, and R_4 are H. Where n and m are 2, and where controlling is enhancement of biofilm formation or enhancement of existing biofilm, X is preferably C_{12} alkyl, R_2 and R_3 are preferably H, and R_1 and R_4 are preferably alkoxycarbonyl. Where n and m are 2, and where controlling is prevention of biofilm formation or dispersion of existing biofilm, preferably, X is C_{12} alkyl, and R_1-R_4 are H.

Applications of control of biofilms

[0040] The present invention provides methods and compounds for controlling biofilms. The method comprises contacting the substrate with a compound described above. In a preferred

embodiment of the invention, the compound and/or composition can be used to prevent biofilm formation by basement molds.

[0045] In an alternative embodiment of the invention, the compound or composition can be used as an antiinfective such as in combination with another antimicrobial, such as an antibiotic. The compound or composition may be used to treat a subject for a disease state associated with biofilm development, such as a bacterial infection, for cystic fibrosis or HIV, or for an immunocompromised subject. Alternatively, the compound and/or composition can be used as a treatment for medical or dental devices such as catheters, tubing, prostheses, etc. to prevent or treat biofilm formation thereon. In an alternative embodiment of the invention, the compound and/or composition can be used in oral care applications such as on teeth or dentures to control plaque and/or odor.

[0046] In an alternative embodiment of the invention, the compound and/or composition can be used to control biofilm formation on skin, e.g., for dandruff control (prevention of *Malassezia* biofilms on scalp), in hand/skin sanitizers (prevention of growth or restoration of natural microflora), for deodorant applications, or for foot care (prevention of fungal growth such as Athletes' Foot without disrupting natural microflora). In an alternative embodiment of the invention, the compound and/or composition can be used in shoe care applications to control bacterial and/or fungal biofilm formation on shoe surfaces. In an alternative embodiment of the invention, the compound and/or composition can be used to prevent toxic shock syndrome or to restore imbalanced microflora (e.g., occluded skin in for example, diapers, or the vaginal tract).

[0047] In an alternative embodiment of the invention, the compound and/or composition can be used in any process machinery having metal, ceramic, glass, composite, or polymer parts, particularly in paper, food, drug, and cosmetic processing applications. The compound and/or composition can be used to prevent biofilm formation on metal, ceramic, glass, composite, or polymer parts and to prevent growth of fungal or bacterial biofilms in paper products.

[0048] In an alternative embodiment of the invention, the compound and/or composition can be used as a food and/or beverage preservative.

[0049] In an alternative embodiment of the invention, the compound and/or composition can be used in generalized surface coatings to prevent biofouling (e.g. paints or coatings for houses, boats,

and route of administration; the age, health, or weight of the subject; the nature and extent of symptoms; the metabolic characteristics of the drug and patient, the kind of concurrent treatment; the frequency of treatment; or the effect desired. Effective doses may be extrapolated from dose response curves from animal model test systems or *in vitro* test systems.

[0055] A dosage unit may comprise diluents, extenders, carriers, liposomes, or the like. The unit may be in solid or gel form such as pills, tablets, capsules and the like or in liquid form suitable for oral, rectal, topical, intravenous injection or parenteral administration or injection into or around the treatment site. The formulations for oral administration may comprise a non-toxic, pharmaceutically acceptable, inert carrier such as lactose, starch, sucrose, glucose, methyl cellulose, magnesium stearate, dicalcium phosphate, calcium sulfate, mannitol, sorbitol, cyclodextrin, cyclodextrin derivatives, or the like. Topical applications for administration according to the method of the present invention include ointments, cream, suspensions, lotions, powder, solutions, pastes, gels, spray, aerosol or oil. Formulations suitable for nasal administration may be administered in a liquid form, for example, nasal spray, nasal drops, or by aerosol administration by nebulizer, including aqueous or oily solutions of the active ingredient. Formulations suitable for parenteral administration include aqueous and non-aqueous formulations isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending systems designed to target the compound to blood components or one or more organs.

[0056] A prodrug refers to a form of the compounds provided herein that has minimal therapeutic activity until it is converted to its desired biologically active form. A prodrug is a compound having one or more functional groups or carriers covalently bound thereto, which functional groups or carriers are removed from the compound by metabolic processes within the body to form the respective bioactive compound. Prodrugs include compounds wherein hydroxy, or amine groups, for example, are bonded to any group that, when administered to a mammalian subject, cleaves to form a free hydroxyl, or amino group, respectively. Examples of prodrugs include, but are not limited to, acetate, formate, or benzoate derivatives of alcohol and amine functional groups, phosphate esters, dimethylglycine esters, aminoalkylbenzyl esters, aminoalkyl esters or carboxyalkyl esters of functional groups.

[0057] A metabolite refers to a break-down or end product of a compound of the present invention or its salt produced by metabolism or biotransformation; e.g., biotransformation to a

ether. Butoxy-propoxy-propanol (BPP) is also contemplated as a solvent. Another type of non-aqueous, low-polarity organic solvent useful herein is the lower molecular weight polyethylene glycols (PEGs). Such materials are those having molecular weights of at least about 150. PEGs of molecular weight ranging from about 200 to 600 are most preferred. Solvents are typically utilized in the present compositions at a level of from about 0% to about 30% by weight of the composition.

[0060] A combination of a 2-alkyl alkanol and a solvent as described herein in a composition incorporated provides acceptable wetting capabilities and thus provides streak-free and evaporation benefits. "Wetting" means that the composition forms a film, instead of single droplets, when applied onto a surface, particularly a hydrophobic surface, and/or does not form droplets on said surface when drying. The formation of droplets during drying can result in visible residues ("streaks") on said surface. By contrast, the drying as a film results in the reduction or even the prevention of visible residues after drying (streak-free cleaning benefit). Furthermore, the wetting capabilities as described herein resulting in the formation of an even film of composition on the surface to which said composition has been applied.

[0061] In alternative embodiments of the invention, the compounds described above may be added to commercially available compositions, such as those available from The Procter & Gamble Company.

[0062] In an alternative embodiment of the invention, the compounds described above can be formulated into cosmetic or pharmaceutical compositions. Suitable adjunct ingredients in topical cosmetic or pharmaceutical compositions include carriers comprising one or more ingredients selected from the group consisting of i) emollients, ii) solvents, iii) humectants, iv) thickeners, v) powders, vi) perfumes, vii) waxes, viii) preservatives, ix) surfactants, x) bases, and others in addition to, or instead of, the adjunct ingredients listed above. Topical compositions that can be applied locally to the skin may be in any form including solutions, oils, creams, ointments, gels, lotions, shampoos, leave-on and rinse-out hair conditioners, milks, cleansers, moisturizers, sprays, skin patches, and the like. Typically, about 1 to about 1000, preferably about 1 to about 100 milligrams per square inch of the composition is applied to the affected area. One of ordinary skill in the art would be able to select appropriate adjunct ingredients and amounts to formulate in the compositions described above without undue experimentation, depending on the compound selected and the intended use of the composition.

[0066] Referring to the apparatus shown in **FIG. 1**, a biofilm may be grown on the surface of disc **4**. This may be accomplished by placing into vial **6** a volume of liquid growth medium inoculated with bacteria or fungi, screwing in cap assembly **7** having positioned therein disc **4**, and placing the vial on a tissue culture rotator in an appropriate controlled temperature environment. Test compounds that are candidates for controlling biofilms can be added to the liquid growth medium before biofilm formation, added after biofilm formation, or both.

Biofilm growth chamber array

[0067] An apparatus for testing biofilm control on a substrate surface is an aspect of the invention. The apparatus comprises a first body having a platform holding a removable substrate surface lacking projections, the substrate surface for growing biofilm thereon; and a second body adapted to receive the first body in alignment, the second body having a plurality of individual wells; wherein the wells are in fluid tight communication with the substrate surface of the first body when the first and second bodies are assembled and in use.

[0068] Referring to **FIG. 2a**, there is shown a side cross sectional view of a re-usable two-piece biofilm growth chamber array (2P-GCA), and shown in **FIG. 2b** is a side cross sectional view of a re-usable three-piece design (3P-GCA). Both the 2P-GCA and the 3P-GCA allow for the growth and testing of biofilms on various materials, under realistic conditions. The illustrations of the parts of the inventions shown in **FIGS. 2a, 2b, 3, and 4** are not meant to be limiting, and certain simple modifications will be obvious to those skilled in the art in light of the present disclosure.

[0069] Referring to the invention depicted in **FIG. 2a**, the 2P-GCA includes a lid assembly **90** that may be configured to accept various materials attached to or part of a growth surface **30**, and a vessel assembly **110**, containing a plurality of individual growth chambers (wells), adapted to provide a fluid tight communication between individual wells **80** of the assembly and surface **30**. Contact between the lid assembly **90** and vessel assembly **110** is maintained by the use of bolts **40** and nuts **45** as well as by the use of alignment pins (made preferably of stainless steel) inserted into alignment pin holes **20**, wherein the fluid tight communications are maintained by the use of individual O-rings **50** (made of a silicone, a synthetic or natural rubber, or other suitable material) by using a single O-ring per well **80**. When the invention is in the fully assembled configuration, O-rings **80** tightly contact both the vessel assembly **110** and the surface **30**.

[0074] Referring to **FIG. 2a** and **FIG. 4**, vessel assembly **110** includes an attachment arm **60** made of polymer or other material, that is adapted to be attached to a tissue culture rotator or similar rotational device able to smoothly rotate the fully assembled growth chamber of the present invention in a temperature controlled environment, as described for the invention disclosed in **FIG. 1**. Such uniform rotation allows for reproducible growth of individual, circular biofilms on the surface of interest **30**.

[0075] When in use, and referring to the 2P-GCA invention shown in **FIG. 2a**, **FIG. 3**, and **FIG. 4**, an array of individual biofilms may be grown on a surface **30** of interest. This may be accomplished by placing O-rings **50** in their seats within vessel assembly **110**, filling the individual wells **80** with the same or different volumes of the same or different liquid growth media, inoculating each well individually or together with the same or different bacteria or fungi, attaching lid assembly **90** that contains the secured surface of interest using alignment pins inserted into the alignment pin holes **20/150**, securing the lid and vessel assemblies together using bolts **40** and nuts **45**, and attaching the entire assembled device on a tissue culture rotator using attachment arm **60** or placing the device with the surface-of-interest side down in a shaking incubator, followed by incubation at the appropriate temperature with or without rotation or shaking, respectively.

[0076] A further apparatus for testing biofilm control on a substrate surface is an aspect of the present invention. The apparatus comprises first and third bodies, each body having a platform holding a removable substrate surface lacking projections, the substrate surface for growing biofilm thereon; and a second body having a first side and a second side, the second body adapted to receive the first and third bodies in sandwich alignment to the first side and second side, the second body having a plurality of individual openings extending from the first side to the second side providing a well open at each end and in fluid tight communication with the substrate surface of the first and third bodies when the apparatus is assembled and in use.

[0077] Referring to **FIG. 2b**, there is shown an alternative embodiment of the vessel assembly invention disclosed in **FIG 2a**. Referring to **FIG. 2b** in addition to **FIG. 3** and **FIG. 4**, it will be seen that the vessel assembly **110** is modified such that the plurality of wells **85** are machined through the entire block **70**, and an additional depression **160**, seats for O-rings **50**, and O-rings **50**

square with a depth of about 3 millimeters added on the reverse side of the block **70** to accommodate a second lid assembly **90**, and the well holes **85** extending entirely through the block.

[0082] When in use, referring to the 3P-GCA invention shown in **FIG. 2b, FIG. 3,** and **FIG. 4,** an array of individual biofilms may be grown on two surfaces **30** of interest, where each of the two surfaces may be made of the same or different material. This may be accomplished in an identical manner to that mentioned above for the 2P-GCA invention, except that the lower lid assembly **90** is attached securely using the threaded bolts **65** and four of the nuts **45** before adding the volumes of liquid growth media.

[0083] In both the 2P-GCA and 3P-GCA embodiments of the invention, test compounds that control biofilms can be added to the liquid growth medium before biofilm formation, added after biofilm formation, or both. Quantification of the effects of such compounds on biofilm formation and/or dispersion is accomplished as described above. After use, surfaces **30** can be removed and discarded or cleaned and re-used, and the wells **80/85** can be easily cleaned for re-use by using a test tube brush and commonly available detergent formulations. This method is particularly suited to the study of biofilms that may be formed on hard surfaces, and their susceptibility to, for example, various cleaning compositions.

[0084] The growth chamber apparatus provided by the present invention is a reusable device where growth and assays are both carried out in the same device. There is no need for physical intervention such as breaking protrusions as in Ceri *et al.* (U.S. 6,326,190 or U.S. 20010049975), or otherwise disturbing the biofilm. The device is optimized for hard, water impermeable surfaces as substrate, however, the device is versatile in that any surface is possible as a substrate. The device is easily configurable for automation with limited or no human intervention in that a robot-removable lid may be used and all steps of addition and removal of liquids, including quantitation steps, may be automated. The device is easily scalable to higher or lower well density and/or larger or smaller wells. Aeration is controllable over a wide range by varying the volume of liquid in the wells, as well as varying the rotation speed of the rotator or shaker.

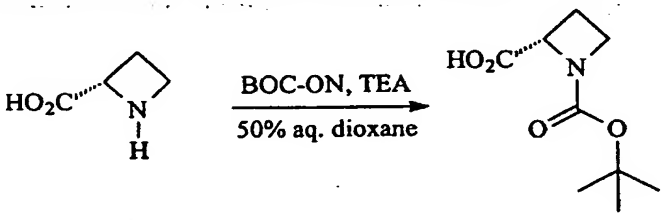
[0085] A method of screening a test compound for control of biofilm on a substrate surface, comprising obtaining the above described apparatus, incubating a biofilm forming microorganism with the test compound in a well of the apparatus under conditions that allow biofilm formation on

Table 1 - Abbreviations

Abbreviations	Definitions
BOC-ON	2-(tert-Butoxycarbonyloxyimino)-2-phenylacetonitrile
CV	crystal violet
DIEA	N,N-Diisopropylethylamine
EtOAc	ethyl acetate
g	grams
ISMS	Ion spray mass spectrometry
mg	milligrams
mL	milliliters
mmol	millimoles
PBS	phosphate buffered saline
PyBOP	Benzotriazol-1-yl-oxytripyrrolidinephosphonium hexafluorophosphate
TEA	Triethylamine
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran

COMPOUND SYNTHESIS EXAMPLES

Example 1 - N-(tert-Butoxycarbonyl)-(S)-(-)-2-azetidinecarboxylic acid (1)



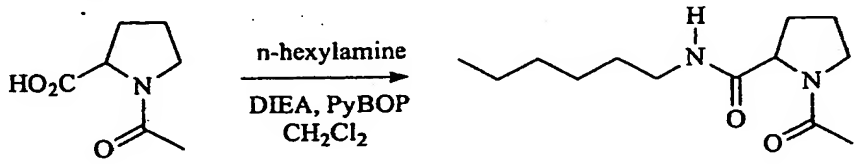
1

[0092] (S)-(-)-2-Azetidinecarboxylic acid (Aldrich Chemical Company, Milwaukee, WI) (1.00 g, 9.89 mmol) is dissolved in 12 mL of 1:1 dioxane:water. Triethylamine (2.1 mL, 14.84 mmol) is added followed by BOC-ON (2.68 g, 10.9 mmol). The mixture is stirred for 6.75 hours then poured onto water (50 mL) and extracted with ether (7 times, 50 mL each). The aqueous solution is cooled in an ice-bath and the pH adjusted to approximately 2.5 with ice-cold 1N HCl solution. The resulting solution is extracted with methylene chloride (3 times, 50 mL each). The combined organic extracts are dried over $MgSO_4$, filtered and concentrated *in vacuo* to afford an oil. Hexane

mL) and poured onto saturated sodium bicarbonate solution. The pH is adjusted to 9 with saturated potassium carbonate solution. The mixture is shaken and the layers separated. The water layer is extracted with methylene chloride (3 times, 5 mL each). The combined organic extracts are washed with water, dried over $MgSO_4$, filtered, and concentrated *in vacuo* affording the desired product (289) as a solid. ISMS: MH^+ 241.2

[0096] Compounds 287, 288, and 290 are prepared in a similar manner from compounds 283, 284 and 286, respectively.

Example 4 - N-(Acetyl)-DL-proline n-decyl amide (7)



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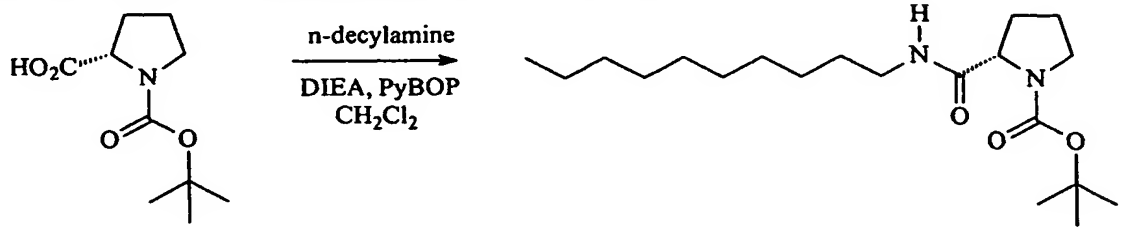
[0097] N-(Acetyl)-DL-proline (Sigma Chemical Company, St. Louis, MI) (100 mg; 0.64 mmol) is dissolved in methylene chloride (3 mL) at ambient temperature. n-Hexylamine (77 mg; 0.76 mmol), N,N-diisopropylethylamine (181 mg; 1.40 mmol) and PyBOP (397 mg; 0.76 mmol) are added sequentially. The reaction is stirred for 18 hours at room temperature, then concentrated under reduced pressure. The residue is purified via silica gel chromatography using a gradient elution (80%→100% ethyl acetate in hexanes, followed by 50%→100% acetone in hexanes) affording the desired product (7). ISMS: MH^+ 241.4

[0098] Compounds 8 and 9 are prepared in a similar manner using n-octylamine and n-decylamine, respectively, in place of n-hexylamine.

Example 5 - N-(tert-Butoxycarbonyl)-L-proline n-decyl amide (12)

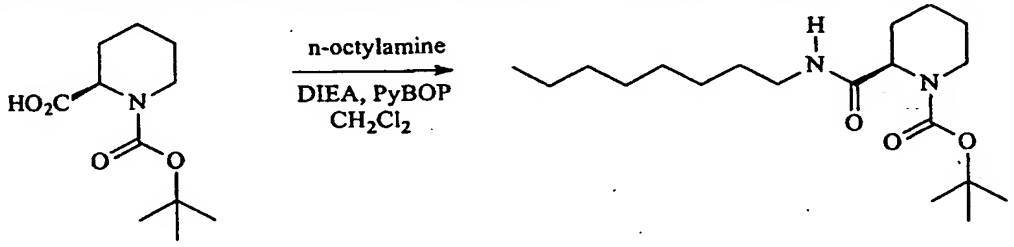


12

[0103] Compounds 30, 31, 165 and 168 are prepared in a similar manner from compounds 10, 11, 155 and 160, respectively.

[0104] Compounds 163, 164, 166, 162 and 167 are prepared in a similar manner from compounds 153, 154, 158, 152 and 159, respectively.

Example 7 - (R)-(+)-1-(tert-Butoxycarbonyl)-2-piperidinecarboxylic acid n-octyl amide (22)

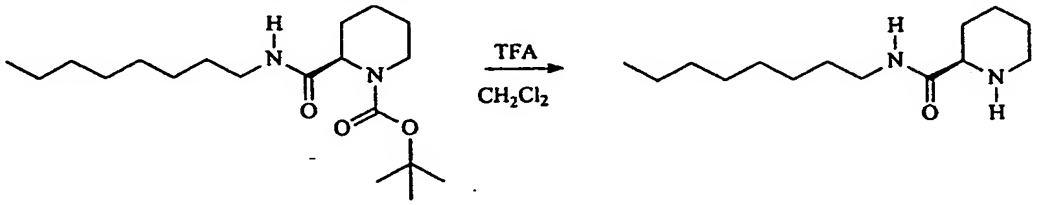


22

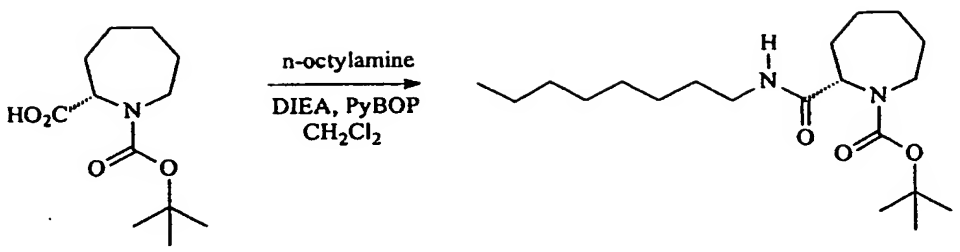
[0105] (R)-(+)-1-(tert-Butoxycarbonyl)-2-piperidinecarboxylic acid (Aldrich Chemical Company, Milwaukee, WI) (1.00 g; 4.36 mmol) is dissolved in methylene chloride (20 mL) at ambient temperature. n-Octylamine (0.620 g; 4.80 mmol), N,N-diisopropylethylamine (1.24 g; 9.60 mmol) and PyBOP (2.50 g; 4.80 mmol) are added sequentially. The reaction is stirred for 25 hours at room temperature, then concentrated under reduced pressure. The residue is purified via silica gel chromatography using a gradient elution (20%→40% ethyl acetate in hexanes) affording the desired product (22) as an oil. ISMS: MH^+ 341.2

[0106] Compound 19 is prepared in similar manner using (S)-(-)-1-(tert-butoxycarbonyl)-2-piperidinecarboxylic acid (Aldrich Chemical Company, Milwaukee, WI) in place of (R)-(+)-1-(tert-butoxycarbonyl)-2-piperidinecarboxylic acid.

Example 8 - (R)-(+)-2-piperidinecarboxylic acid n-octyl amide (23)



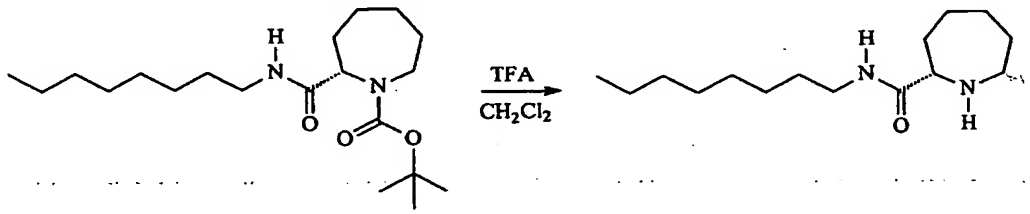
22 23



2

[0111] (S)-(-)-1-(tert-Butoxycarbonyl)-2-azepanecarboxylic acid (prepared as described in *Tetrahedron Letters* (1994), 35(2), 237-240) (1.00 g; 4.11 mmol) is dissolved in methylene chloride (20 mL) at ambient temperature. n-Octylamine (0.584 g; 4.52 mmol), N,N-diisopropylethylamine (1.17 g; 9.04 mmol) and PyBOP (2.35 g; 4.52 mmol) are added sequentially. The reaction is stirred for 24 hours at room temperature, then concentrated under reduced pressure. The residue is purified via silica gel chromatography affording the desired product (2).

Example 11 - (S)-(-)-2-azepanecarboxylic acid n-octyl amide (3)



2 3

[0112] (S)-(-)-1-(tert-Butoxycarbonyl)-2-azepanecarboxylic acid n-octyl amide (2) (1.00 g; 2.82 mmol) is dissolved in methylene chloride (40 mL) at ambient temperature. Trifluoroacetic acid (20 mL) is added and the solution is stirred for 7 hours at ambient temperature. The solution is concentrated *in vacuo* at 40°C. The residue is dissolved in methylene chloride (200 mL) and poured onto saturated sodium bicarbonate solution. The pH is adjusted to 9 with saturated potassium carbonate solution. The mixture is shaken and the layers separated. The water layer is extracted with methylene chloride (3 times, 50 mL each). The combined organic extracts are washed with water, dried over $MgSO_4$, filtered, and concentrated *in vacuo* affording the desired product (3).

Example 12 - N-(tert-Butoxycarbonyl)-D-proline 1,12-diaminododecane bisamide (225)

Primary, High-Throughput, Microplate Screening Methods

[0115] The high-throughput screening methods described are based on the ability of bacteria to adhere to and form biofilms on 96-well microplates made of polystyrene (PS), polyvinyl chloride (PVC), polypropylene (PP), or other materials, and on their ability to be stained with a dye such as crystal violet (CV) that stains the biofilms but does not stain the plastic, followed by ethanolic extraction of the dye and spectrophotometric quantitation using a microplate reader.

[0116] Various versions of such screens have been well described for several bacterial species (see, for example, Cowan, M. M., and Fletcher, M., 1987, *J. Microbiol. Methods* 7:241-249; Shea, C., and Williamson, J. C., 1990, *Biotechniques* 8:610-611; O'Toole, G. A., and Kolter, R., 1998, *Mol. Microbiol.* 28:449-461; Loo, C. Y. *et al.*, 2000, *J. Bacteriol.* 182:1374-1382); almost any bacterium can be used. The screens can also be modified for similar use with fungi such as yeasts, by choosing optimal dyes and growth media.

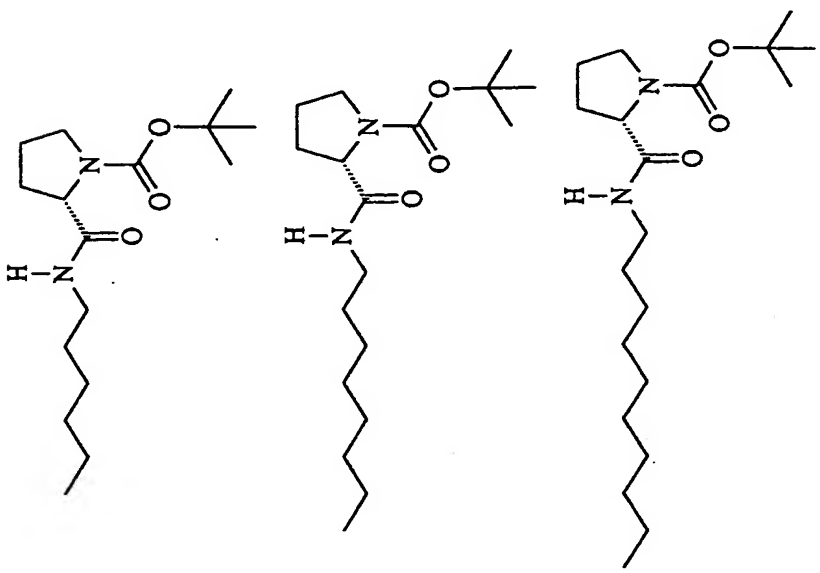
[0117] This invention relates to improved screens that are easy, rapid, and are designed to facilitate automation. They can be used to discover compounds that inhibit or enhance the formation of biofilms, or additionally to discover compounds that initiate dispersion of biofilms. The screening methods can also be used to determine the susceptibility of bacteria present in biofilms to biocides or other antimicrobials.

Reference Example 1 - Biofilm prevention screen to screen for compounds that inhibit formation of biofilms.

[0118] Overnight cultures of 3 mL are made of *P. aeruginosa* strain PAO1 (grown in R2A medium (*Handbook of Microbiological Media*; CRC Press, 1997, second edition, R. M. Atlas, ed.), modified to omit Mg^{2+} but to include 0.3-0.5% fructose (pH 7.0) and 3 μM ferrous ammonium sulfate), *P. fluorescens* strain ATCC 13525 (grown in R2A modified to omit Mg^{2+} but to include 0.3-0.5% sodium pyruvate (pH 7.0)), *S. epidermidis* strain ATCC 35984 (grown in a ¼ dilution of TSB (*Handbook of Microbiological Media*; CRC Press, 1997, second edition, R. M. Atlas, ed.) supplemented with 1% fructose (pH 7.0) and 3 μM ferrous ammonium sulfate). To each type of growth medium, HEPES (pH 7.3) is optionally added to a final concentration of 10 mM.

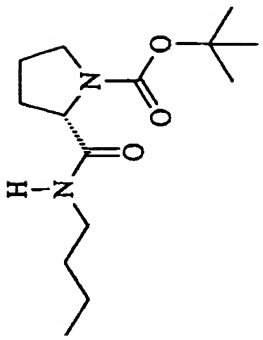
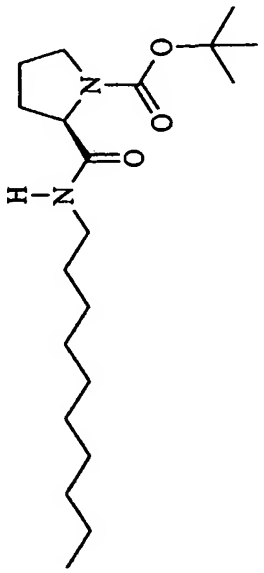
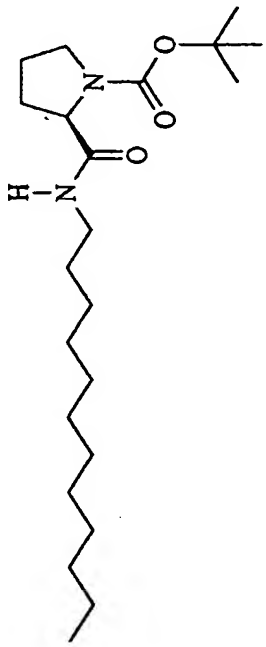
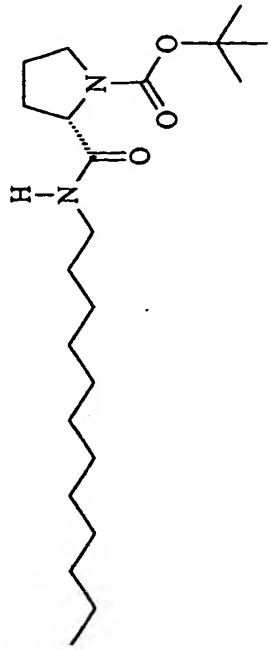
[0119] To PS, PVC, or PP (preferably PP or PVC) round-bottom, 96-well microplates containing 100μl per well of the respective growth medium as described above, compounds to be

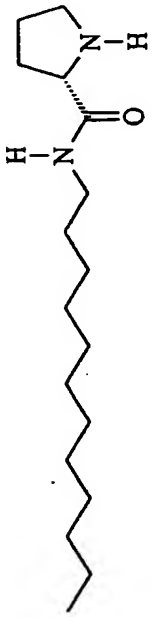
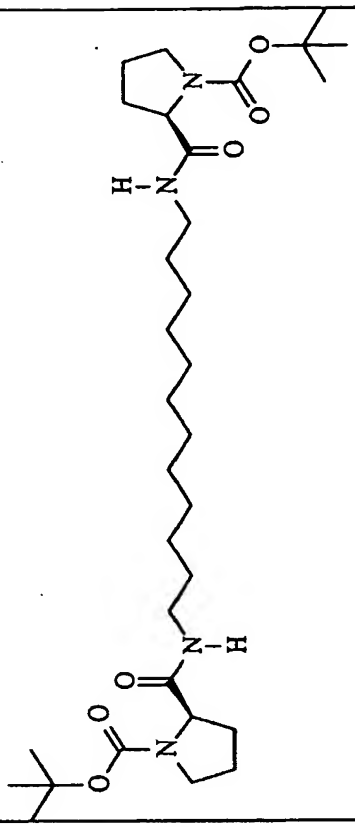
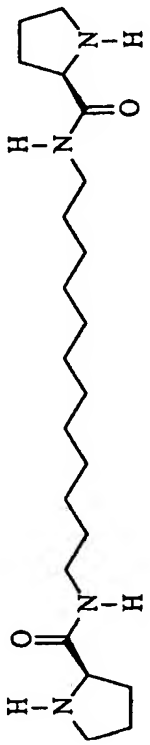
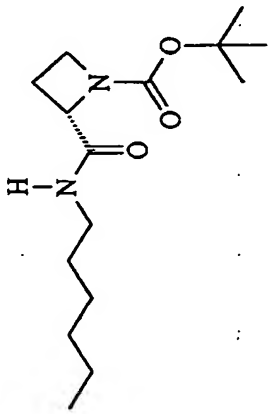
10%). This table clearly shows the efficacy of certain compounds in the prevention of biofilm formation on one or more bacterial species.



10	-16%	-16%	-7%	-6%	-5%	-1%	-25%	-17%	-18%
11	-15%	-18%	-6%	-26%	-6%	-4%	37%	-60%	10%
12	-32%	-45%	6%	-37%	-42%	-16%	31%	-2%	-21%

23	-3%	-31%	-5%	21%	-13%	-23%	-36%	15%	-1%					
24	-11%	-22%	-12%	-2%	-6%	-1%	-53%	-20%	-8%					
28	-9%	-3%	0%	26%	38%	21%	-51%	-44%	-5%					
30	18%	9%	2%	19%	11%	11%	-6%	-7%	-12%					
31	-44%	-125%	-37%	52%	5%	3%	-27%	-1%	-7%					

	155	6%	2%	-5%	4%	4%	10%	21%	-1%	-3%
	158	-40%	-34%	1%	-40%	-11%	3%	54%	52%	-10%
	159	-50%	-38%	-9%	-13%	-3%	-1%	-3%	-20%	-9%
	160	-59%	-44%	-2%	-18%	0%	-1%	29%	20%	-9%

	168	91%	53%	3%	93%	97%	26%	98%	100%	101%
	225	-8%	7%	11%	-14%	0%	14%	-39%	-24%	-21%
	226	95%	96%	-23%	69%	10%	-19%	100%	101%	24%
	283	0%	-10%	-7%	13%	-1%	3%	-23%	-10%	-3%

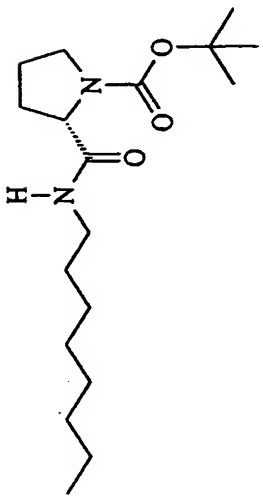
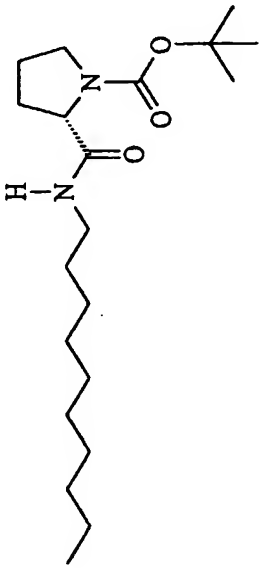
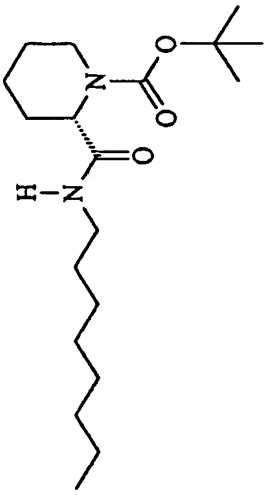
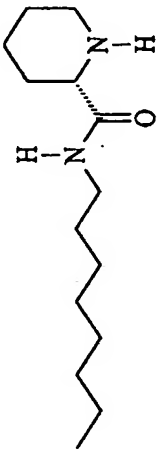
288	96%	97%	12%	59%	40%	-14%	99%	101%	101%
289	77%	-2%	9%	99%	45%	-8%	101%	101%	39%
290	37%	-16%	6%	93%	98%	-12%	97%	100%	101%

to the inoculation of bacteria, and growth in the duplicate plates for 24 hours is allowed using the conditions specified in Reference Example 1.

[0126] After growth of biofilm to be tested in the wells, cells present in biofilm attached to one of the duplicate microplates is stained with CV, quantitated as described in Reference Example 1, and the data for the entire plate averaged, resulting in a growth control value, t_0. The remaining replicate of each microplate pairs is treated with compounds in DMSO, or DMSO alone for control wells, by first removing the cell suspensions from the wells, then adding fresh 100 μL volumes of the same appropriate liquid growth media, followed by direct addition and mixing of the compound DMSO solutions to give final compound concentrations of 390 μM. Incubation of the compound-containing microplates is then continued for an additional 22-26 hours using the same conditions described in Reference Example 1. Cell suspensions are then removed, 100 μL per well of a 0 mM NaOH (*S. epidermidis*), 20 mM NaOH (*P. fluorescens*), or 30 mM NaOH (*P. aeruginosa*) solution in PBS is added, and incubation at room temperature is allowed for 30 minutes. No addition of NaOH is made to the *S. epidermidis* suspension since a test assay indicated no amplification by base with *S. epidermidis*. The microplates are subsequently rinsed three times with 150μL per well of PBS, followed by final liquid removal, staining with CV and quantitation as described in Reference Example 1. Data for each well that contained compound result in a compound value, R, and data for control wells that contained DMSO only are averaged to generate a second control value, t_{24}. The effect of each compound tested on dispersion of the biofilm compared to the two control values is calculated thus:

$$\text{If } R < t_0, \text{ then } \%\,\text{Reduction} = \frac{t_0 - R}{t_0}; \text{ and if } R > t_{24}, \text{ then } \%\,\text{Reduction} = \frac{t_{24} - R}{t_{24}}.$$

[0127] Table 3 shows the results of this biofilm dispersion screen carried out using the compounds described herein, run against biofilms of the three bacterial species specified in Reference Example 1. Data are expressed in terms of the average percentage reduction of biofilm formed in the presence of each compound, relative to the two control values, for quadruplicate samples. Assays are run as described above, using compound concentrations of 390 μM. Positive numbers indicate dispersion (reduction) of pre-existing biofilms by a compound, and negative numbers indicate enhancement (stimulation of growth) of pre-existing biofilms by a compound (percentage coefficients of variation are ≤ 15%). This table clearly shows the efficacy of certain compounds in the dispersion of pre-existing biofilms on one or more bacterial species.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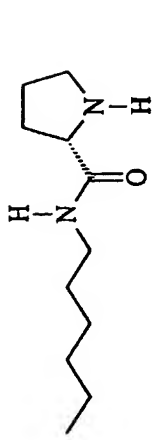
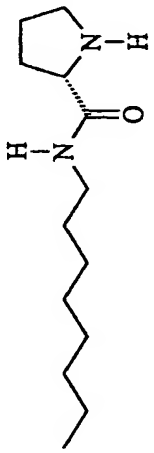
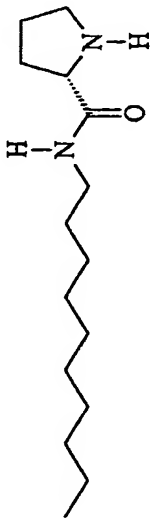
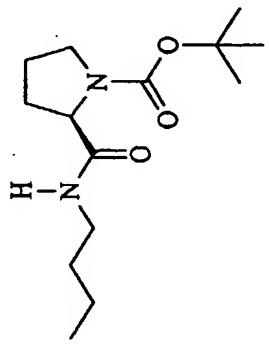
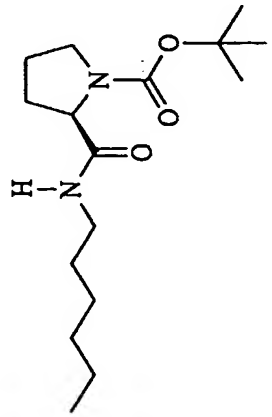
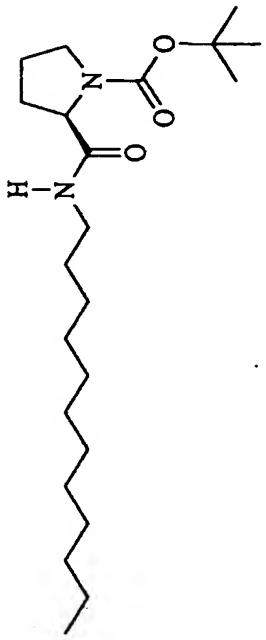
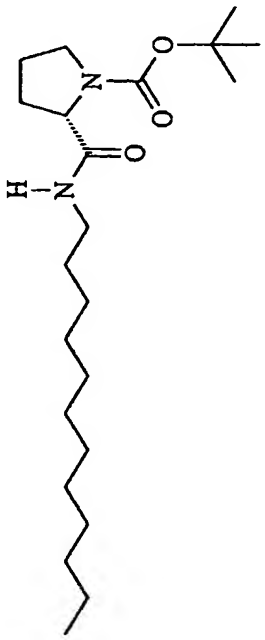
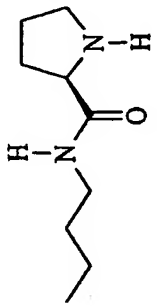
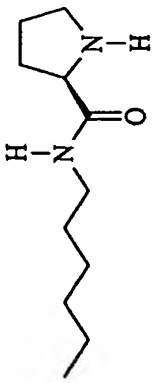
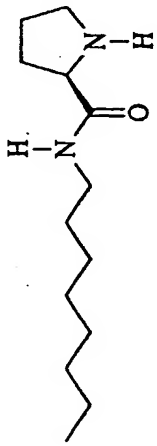
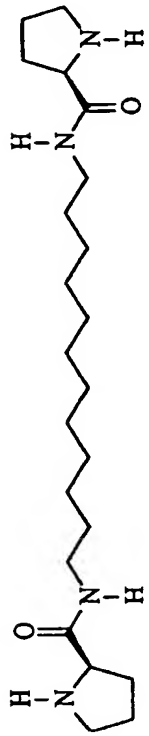
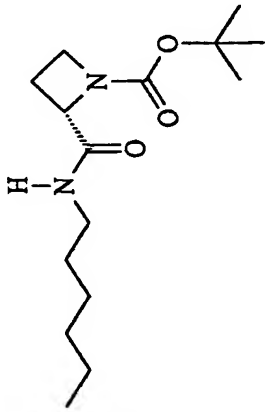
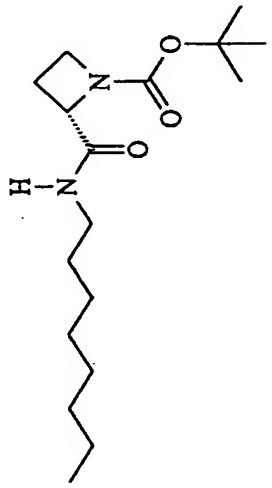
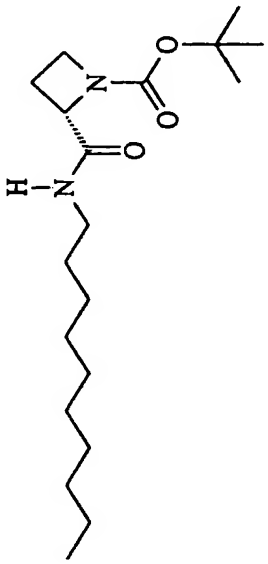
-23%	-30%	0%	25%
-56%	-30%	-25%	46%
11	12	19	20

30		1%	-107%	72%	-11%
31		21%	-9%	1%	-4%
32		0%	-7%	32%	-11%
152		1%	1%	20%	-10%
153					

	159	-55%	-94%	0%	
	160	-52%	-44%	0%	
	162	-23%	0%	0%	
	163	4%	12%	-5%	
	164	38%	47%	-3%	

	226	7%	32%	26%	
	283	-11%	0%	-11%	
	284	-79%	-15%	0%	
	285	-37%	-74%	0%	

Screening Models for Biofilms Grown on Various Hard Surfaces

[0128] Quantitative methods for growing model biofilms on any hard surface have been developed, for purposes of testing the efficacy of compounds that control biofilms, such as those discoverable using Reference Example 1. The methods can also be used to determine the susceptibility of bacteria present in biofilms to biocides or other antimicrobials, and to determine the efficacy of compounds that initiate dispersion of biofilms, such as those discoverable using Reference Example 2. Methods to grow and test defined biofilms on surfaces have been previously described (see for example, U.S. Patent Nos. 6,051,423; 5,605,836; 6,326,190; 2001/0049975; WO0077162; and EP 1038972), however, these methods do not allow for application to many different surfaces, are not amenable to a high-throughput mode, or are not easily made quantitative, or have other features that limit their broad implementation.

[0129] This invention relates to methods that can be applied to biofilm growth of almost any bacterium on almost any surface (including polymers, ceramics, metals, glasses, composites, and natural surfaces such as woods), are quantitative, reproducible, and can be made high-throughput. The methods of this invention can also be modified for similar use with fungi such as yeasts, by choosing optimal dyes and growth media. Additionally, the methods of this invention can be used with a consortium of native microbial inocula taken from a biofilm(s) present in a real-world setting or natural environment such as a shower tile surface, toilet bowl surface, skin surface, water pipe, dental or medical waterlines, soil sample, and the like.

Reference Example 4 - Biofilm growth and test method based on growth in individual plastic vials

[0130] Polypropylene 2 ml cryogenic storage vials (Corning #2027) with an internal threaded cap and O-ring are modified by inserting 8 millimeter discs of shower tile, shower-stall acrylic plastic, or toilet bowl porcelain into the cavity of the cap. Discs are fixed in place to be flush with the top of the inside cap either by making the diameter slightly larger than the plastic cap, or by first applying a standard silicone adhesive in the cap cavity. The net result is a uniform surface on which a biofilm can be grown, as depicted in **FIG. 1**.

[0131] Modified vials are filled with 0.5-1 mL of modified R2A medium with fructose or pyruvate supplementation (as described in Reference Example 1), and inoculated with 5-10 µL of overnight cultures of *P. aeruginosa* strain ATCC 10145 or *P. fluorescens* strain ATCC 13525. Inoculated vials are then placed on a tissue culture rotator (VWR Scientific) at 20-25^{0}C, set at a

TABLE 4

Vial #	$OD_{586\ nm}$
1	0.936
2	0.840
3	0.601
4	0.895
5	0.881
6	0.794
control 1	0.076
control 2	0.085
blank	0.038
blank	0.037

Reference Example 5 - Biofilm growth and test method based on 2-piece growth-chamber array

[0134] A polypropylene block is machined as indicated in **FIGS. 2a, 3, and 4**, to generate an array of 24 individual, cylindrical wells that essentially mimic 24 of the cryogenic storage vial bottoms described in Reference Example 4 ('block bottom'). Each well is machined to provide a seat for an O-ring or gasket at the top, and the well-to-well spacing is designed to match that of a standard 96-well microplate, so that a multi-channel pipettor could be used for liquid manipulations. A separate block of polypropylene is machined to provide a recessed lid ('block top') to accommodate a 108 x 108 millimeter surface (e.g. bathroom tile, porcelain, plastic, glass), as illustrated in **FIGS. 2a, 3, and 4**. The block bottom contains metal pins to align the block top and bottom, while the block top contains holes drilled to accommodate the pins from the bottom block. The block top also contains set screws to clamp the surface into the recessed holding platform. Additionally, the block bottom contains an adapting arm to allow it to fit onto the motorized portion of a tissue culture rotator. With the surface attached to the top block, the top block is then aligned with the bottom, such that the surface contacts the O-rings or gaskets, which provides a seal to the surface. Threaded holes are machined into both top and bottom blocks to accommodate screws that are used to securely clamp the top and bottom blocks together to provide sufficient pressure on the O-rings to generate the seals. The whole assembly thus accurately mimics 24 of the vial-cap assemblies, as described in Reference Example 3. Optionally, the block

[0138] It will be obvious to one skilled in the art in light of the present disclosure that the present growth and test method, which is described to test for compounds that prevent biofilm formation, can easily be applied to screening for compounds that initiate dispersion of biofilms, by simply allowing biofilm growth first in the absence of compounds, then measuring the effects of their subsequent addition.

[0139] Table 5 shows data collected using the test method described, with biofilm growth of *P. fluorescens* on standard white matte bathroom tile continued for 72 hours, including two exchanges of the liquid growth medium. A polypropylene block top and bottom are used. Data are reported as optical density measured at 586 nm for 200 μL of extracted dye solution, for 16 replicates of inoculated wells, and 8 control well replicates. Control wells (columns 5 and 6) are not inoculated with bacteria, but are treated exactly as for inoculated wells (columns 1 to 4). Smaller numbers indicate less biofilm growth.

TABLE 5

	1	2	3	4	5	6
A	0.442	0.444	0.576	0.566	0.302	0.331
B	0.463	0.412	0.538	0.603	0.282	*
C	0.451	0.358	0.488	0.623	0.267	0.324
D	0.478	0.579	0.624	0.691	0.260	0.304

* no data obtainable

[0140] Table 6 shows similar optical density data, also collected using the test method described, but for biofilm growth of *S. epidermidis* on standard white glossy bathroom tile, for 72 hours, including two exchanges of the liquid growth medium. A polypropylene block top and bottom are used. Data are reported as optical density measurements for 20 replicates of inoculated wells (columns 1 to 5), and 4 control well replicates (column 6). Smaller numbers indicate less biofilm growth.

TABLE 6

	1	2	3	4	5	6
A	0.830	0.595	1.065	1.152	1.111	0.241
B	0.633	0.858	0.849	0.878	0.743	*
C	0.842	0.746	1.561	0.822	0.801	0.167

(compound 32), 2 (compound 168), 3 (compound 226), 4 (inoculated control), and 5 (uninoculated control). Smaller numbers indicate prevention of biofilm growth.

TABLE 7

	1	2	3	4	5
A	0.175	1.981	0.202	2.387	0.211
B	0.244	1.581	0.269	1.870	0.162
C	0.207	1.517	0.147	2.201	0.206

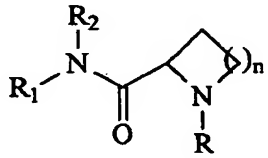
[0146] From this data, compounds 32 and 226 clearly have a strong biofilm prevention activity, whereas compound 168 has a much more modest effect in this test method, the method being significantly more realistic but lower throughput that the methods described in Reference Examples 1 and 2.

[0147] It will be obvious to one skilled in the art in light of the present disclosure that the present growth and test method, which is described to test for compounds that prevent biofilm formation, can easily be applied to screening for compounds that initiate dispersion of biofilms, by simply allowing biofilm growth first in the absence of compounds, then measuring the effects of their subsequent addition.

[0148] While particular embodiments of the present invention have been illustrated and described, it would be obvious to those skilled in the art in light of the present disclosure that various other changes and modifications can be made without departing from the spirit and scope of the invention. It is therefore intended to cover in the appended claims all such changes and modifications that are within the scope of this invention.

What is claimed is:

1. A method of controlling biofilm on a substrate, comprising:
contacting the substrate with a compound having structure A:



A

wherein n is 1-4; and

when n is 1,

R is H, alkyl, acyl, or alkoxycarbonyl; and

R_1 and R_2 are independently H, C_6-C_{20} alkyl or alkenyl;

wherein when R is H, one of R_1 and R_2 is other than H;

when n is 2,

R is H, alkyl, acyl, or alkoxycarbonyl; and

R_1 and R_2 are independently H; C_4-C_{20} alkyl or alkenyl;

wherein when R is H, one of R_1 and R_2 is other than H;

when n is 3,

R is H, alkyl, acyl, or alkoxycarbonyl; and

R_1 and R_2 are independently H; C_5-C_{20} alkyl or alkenyl;

wherein when R is H, one of R_1 and R_2 is other than H; or

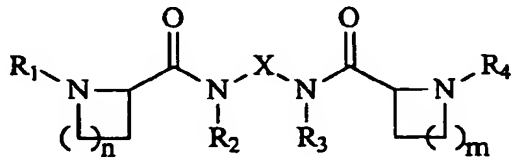
when n is 4,

R is H, alkyl, acyl, or alkoxycarbonyl; and

R_1 and R_2 are independently H; C_1-C_{20} alkyl or C_2-C_{20} alkenyl;

or a salt thereof, or a stereoisomer thereof;

or contacting the biofilm with a compound having structure B:



B

wherein

n and m are independently 1, 2, 3, or 4,

X is C_1-C_{20} alkyl, or C_2-C_{20} alkenyl;

R_1 and R_4 are independently H, alkyl, acyl or alkoxycarbonyl, and

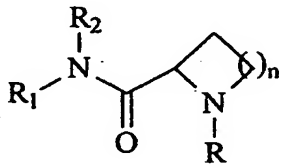
R_2 and R_3 are independently H, or alkyl;

with the proviso that when n and m are 2, X is C_8-C_{20} alkyl, or C_8-C_{20} alkenyl;

or a salt thereof, or a stereoisomer thereof;

for a time sufficient to control the biofilm on the substrate.

2. A compound having structure A:



A

wherein n is 1, 3, or 4; and

when n is 1,

R is H, alkyl, acyl, or alkoxycarbonyl; and

R_1 and R_2 are independently H, C_6-C_{20} alkyl or alkenyl;

wherein when R is H, one of R_1 and R_2 is other than H;

when n is 3,

R is H, alkyl, acyl, or alkoxycarbonyl; and

R_1 and R_2 are independently H; C_5-C_{20} alkyl or alkenyl;

wherein when R is H, one of R_1 and R_2 is other than H; or

when n is 4,

R is H, alkyl, acyl, or alkoxycarbonyl; and

wherein the wells are in fluid tight communication with the substrate surface of the first body when the first and second bodies are assembled and in use.

7. An apparatus for testing biofilm control on a substrate surface, the apparatus comprising:

first and third bodies, each body having a platform holding a removable substrate surface lacking projections, the substrate surface for growing biofilm thereon; and

a second body having a first side and a second side, the second body adapted to receive the first and third bodies in sandwich alignment to the first side and second side, the second body having a plurality of individual openings extending from the first side to the second side providing a well open at each end and in fluid tight communication with the substrate surface of the first and third bodies when the apparatus is assembled and in use.

8. A method of screening a test compound for control of biofilm on a substrate surface, comprising:

obtaining the apparatus of Claim 7;

incubating a biofilm forming microorganism with the test compound in a well of the apparatus under conditions that allow biofilm formation on the substrate surface absent the test compound;

comparing biofilm formation on the substrate surface in the presence of the test compound with biofilm formation on the substrate surface in the absence of the test compound,

wherein when biofilm formation in the presence of the compound is less than in the absence of the test compound, then the test compound has inhibitory activity for biofilm formation on the substrate surface, and

wherein when biofilm formation in the presence of the compound is greater than in the absence of the test compound, then the test compound has stimulatory activity for biofilm formation on the substrate surface.

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 02/13028

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12M1/34 A01N43/34 A01N43/36 A01N43/40 A01N47/16
C07D205/04 C07D207/16 C07D211/60 C07D223/06

According to International Patent Classification (IPC) or to both national classification and IPC.

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12M A01N C07D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BEILSTEIN Data, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 99 43658 A (RYDER TODD ROBERT ;WARNER LAMBERT CO (US); HU LAIN YEN (US); RAFFE) 2 September 1999 (1999-09-02) claims	2-5
A	EP 0 208 279 A (SEARLE & CO) 14 January 1987 (1987-01-14) claims; examples	2-5
A	WO 00 06177 A (UNIV IOWA RES FOUND) 10 February 2000 (2000-02-10) cited in the application claims	1-5,8-10

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 September 2002	26/09/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Chouly, J

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INTERNATIONAL SEARCH REPORT

ormation on patent family members

Intes nal Application No
PCT/US 02/13028

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9943658 A	02-09-1999	AU 1600599 A	15-09-1999
		WO 9943658 A1	02-09-1999
		US 6251919 B1	26-06-2001
EP 0208279 A	14-01-1987	US 4649147 A	10-03-1987
		AU 583400 B2	27-04-1989
		AU 5974586 A	15-01-1987
		CA 1276152 A1	13-11-1990
		DE 3665582 D1	19-10-1989
		EP 0208279 A1	14-01-1987
		JP 2015097 C	02-02-1996
		JP 7051559 B	05-06-1995
		JP 62010061 A	19-01-1987
		ZA 8605026 A	30-09-1987
WO 0006177 A	10-02-2000	AU 5544999 A	21-02-2000
		EP 1100513 A1	23-05-2001
		WO 0006177 A1	10-02-2000
WO 0032152 A	08-06-2000	AU 1933800 A	19-06-2000
		EP 1135144 A1	26-09-2001
		WO 0032152 A2	08-06-2000
		US 2002072052 A1	13-06-2002
WO 9807883 A	26-02-1998	FI 963235 A	17-02-1998
		EP 0879295 A1	25-11-1998
		WO 9807883 A1	26-02-1998
		JP 11513901 T	30-11-1999
		NO 981691 A	15-04-1998
		US 5910420 A	08-06-1999
WO 9733972 A	18-09-1997	AU 724282 B2	14-09-2000
		AU 2088797 A	01-10-1997
		CA 2248808 A1	18-09-1997
		WO 9733972 A1	18-09-1997
		DE 29723730 U1	04-03-1999
		EP 0904347 A1	31-03-1999
		JP 2000506021 T	23-05-2000
		US 6051423 A	18-04-2000
		US 6410256 B1	25-06-2002
		US 6326190 B1	04-12-2001

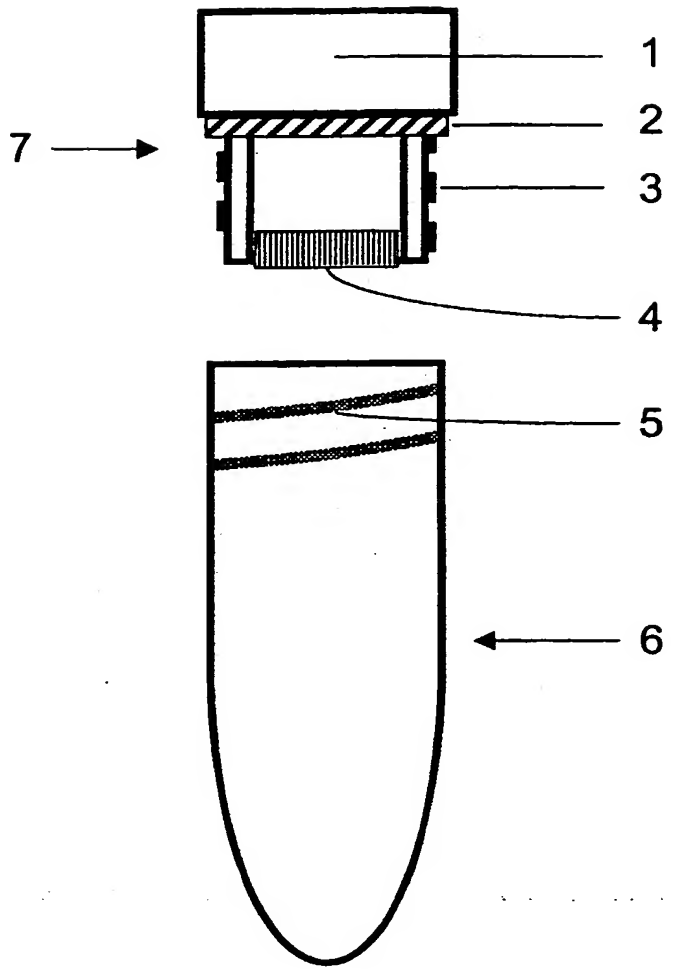

FIG. 1

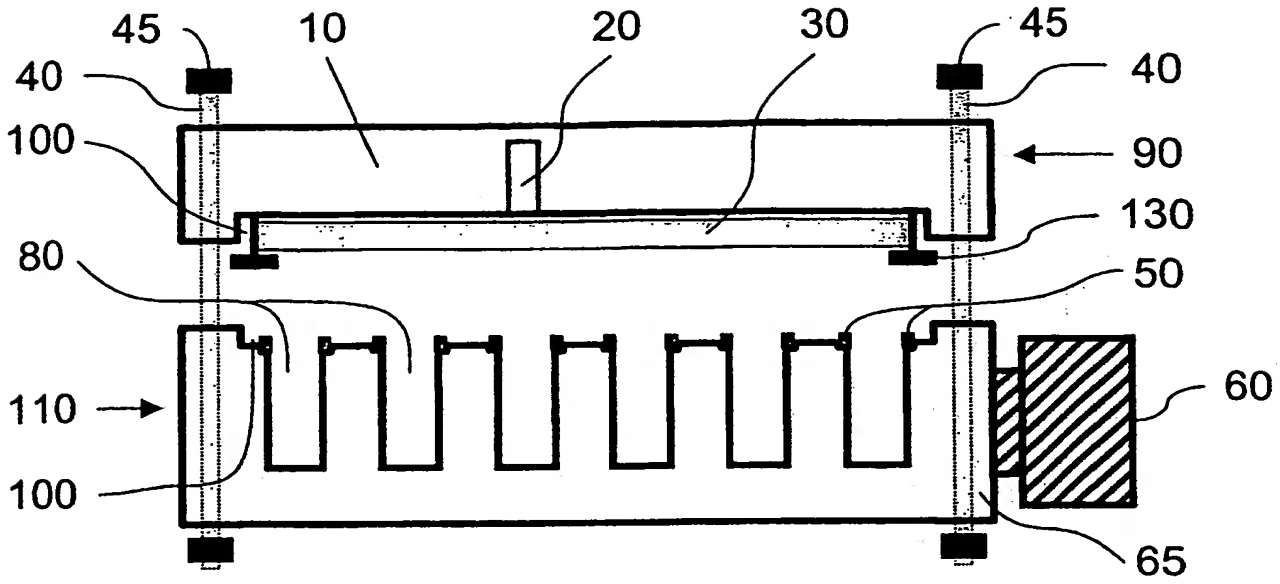
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FIG. 2a

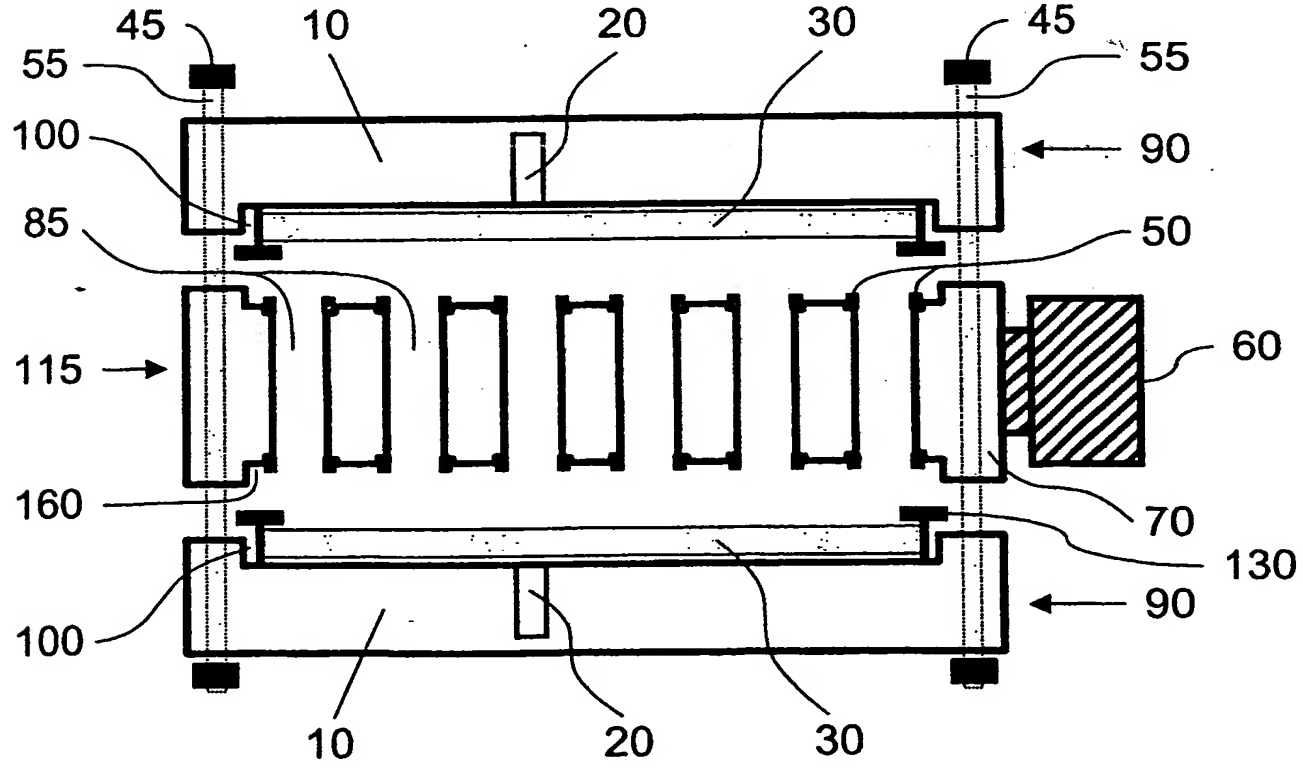
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FIG. 2b

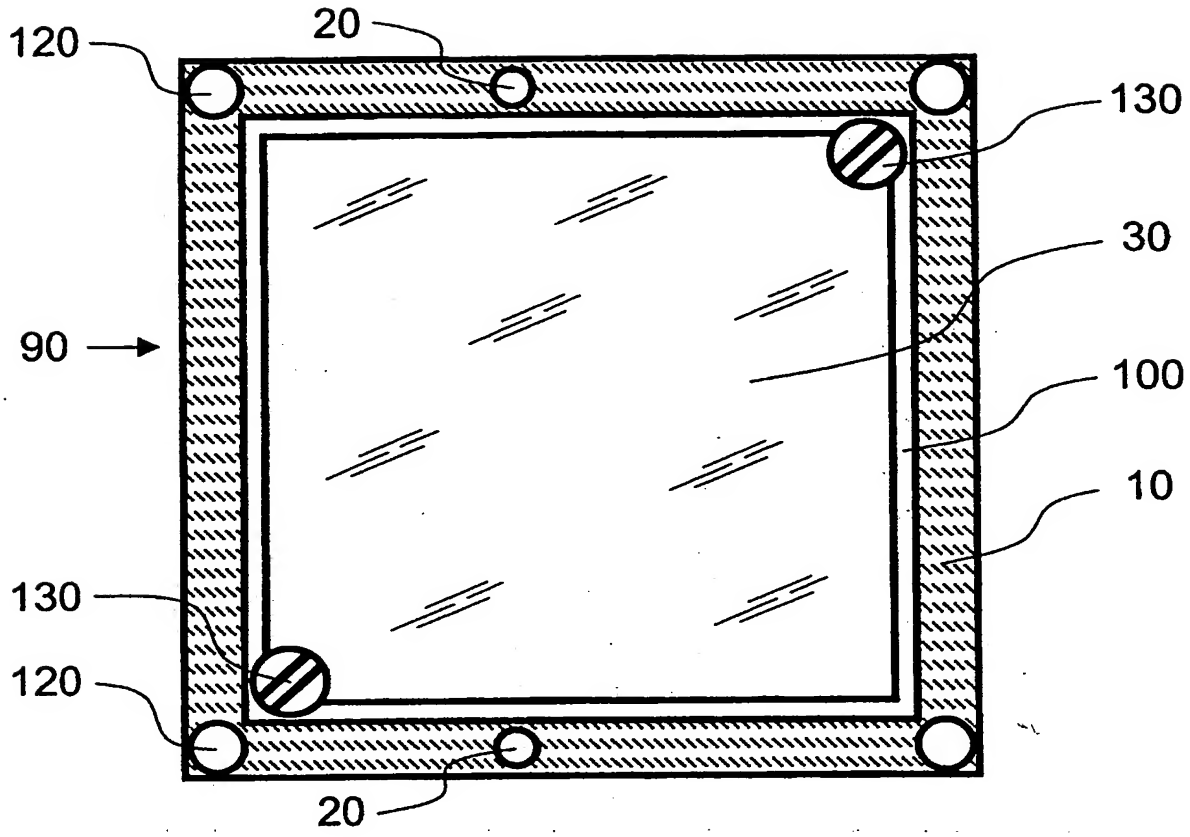

FIG. 3

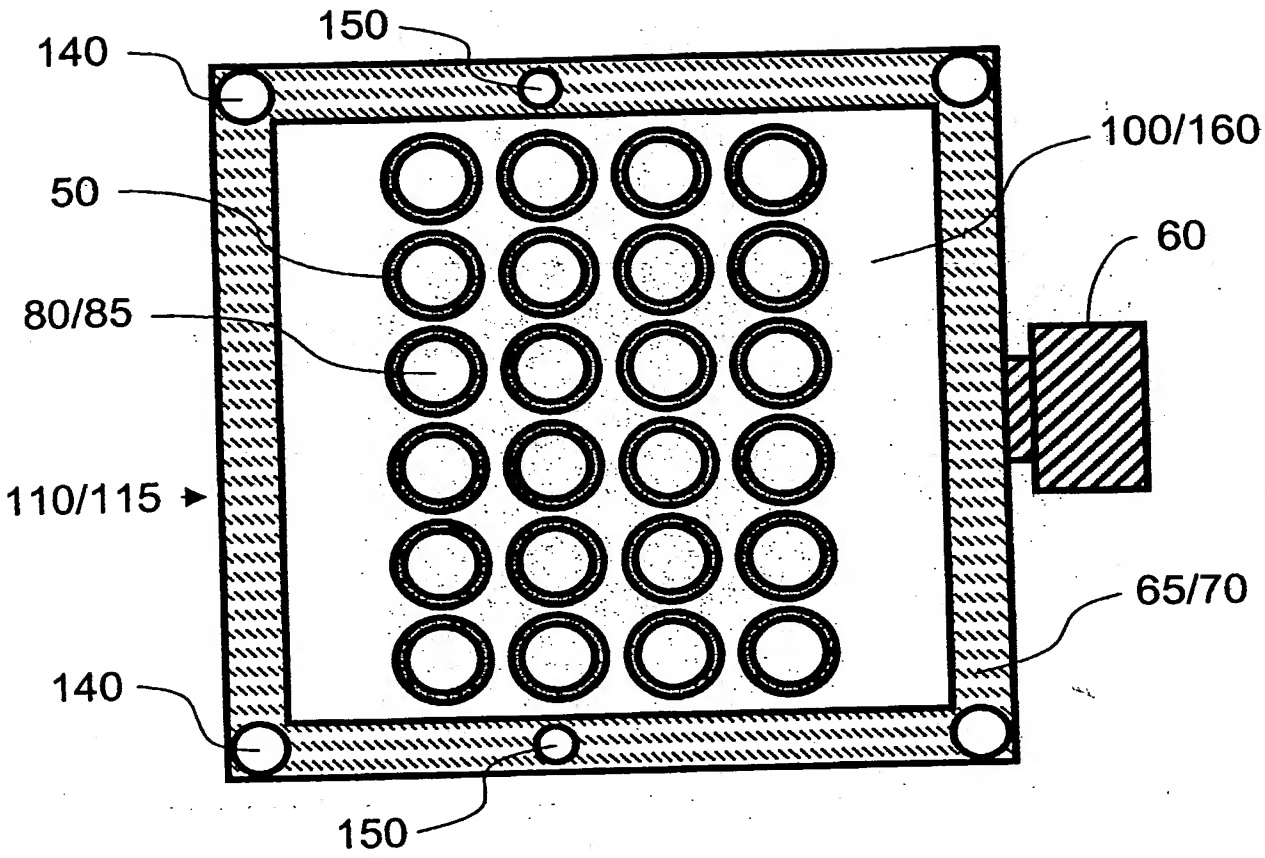

FIG. 4